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RNA Transport to the Vegetal Cortex of *Xenopus* Oocytes

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Xcat-2 RNA, a component of the germ plasm in *Xenopus*, localizes with the mitochondrial cloud material to the vegetal cortex in stage II oocytes. *Vg*1 RNA also localizes to the vegetal cortex, but later in stage III/IV oocytes, using a microtubule dependent pathway. To further analyze the mechanisms involved in RNA transport, *in situ* hybridization and autoradiography were used to follow the localization of endogenous *Vg*1 and injected *Xcat*-2 transcripts in stage IV oocytes. We show that *Xcat*-2 is competent to localize to the vegetal cortex quite independently of the mitochondrial cloud. *Xcat*-2 RNA appears to use the late *Vg*1 localization pathway, as depolymerization of microtubules by cold or nocodazole treatment prevented translocation of *Xcat*-2 transcripts, but did not result in the disruption of *Xcat*-2 anchored in the cortex. Furthermore, RNA transport was shown to be stage dependent for both *Vg*1 and *Xcat*-2 RNAs, as they did not localize in fully grown stage VI oocytes after injection. RNA sequences both required and sufficient to direct *Xcat*-2 to the vegetal cortex were mapped to a sequence of 150 nt immediately adjacent to the open reading frame and additional sequences at the end of the 3′ untranslated region. Mapping was accomplished by injecting deletion mutant transcripts into stage IV oocytes and monitoring localization by RNase protection and autoradiography. All mutants competent for translocation were also capable of cortical anchoring, suggesting that the same signal is used for both steps. We speculate that two separate RNA pathways evolved during the course of *Xenopus* oogenesis. One pathway, specialized for the transport of germ plasm by way of the mitochondrial cloud, occurs early to ensure the segregation of the germ cell lineage. The other, late, pathway may serve as the more general transport system for localizing RNAs involved in somatic cell differentiation.

INTRODUCTION

Targeting of proteins to different subcellular domains is an integral if not primary step in generating a polarized cell. A new strategy for asymmetrically distributing cytoplasmic proteins became evident with the discovery of mRNAs that accumulate to either pole of the *Xenopus* (King and Barklis, 1985; Rebagliati *et al.*, 1985; Mosquera *et al.*, 1993) or *Drosophila* oocyte (Lehmann and Nusslein-Volhard, 1986; Driever and Nusslein-Volhard, 1988a, b). The consequences of RNA localization for development are profound as local protein gradients are generated that can initiate pattern formation in the early embryo (Wang and Lehmann, 1991). Localized mRNAs have also been described in a variety of somatic cells including fibroblasts (Kislauskis *et al.*, 1994), myoblasts (Cripe *et al.*, 1993), neurons (Garner *et al.*, 1988; Bruckenstein *et al.*, 1990), and oligodendrocytes (Trapp *et al.*, 1987). Here, such localized proteins affect cell morphology and function. Therefore, it appears that RNA targeting is an important regulatory mechanism for localizing proteins to subcellular domains in somatic cells as well as germ cells (Wilhelm and Vale, 1993; St. Johnston, 1995; King, 1995).

The vegetal cortex of *Xenopus* oocytes functions as a special domain for receiving and anchoring localized mRNAs including *Vg*1, *Xcat*-2 (Elinson *et al.*, 1993), and *Xwnt*11 (Ku and Melton, 1993). These RNAs encode proteins that are likely to be involved in pattern formation or germ cell specification. *Vg*1 and *Xwnt*11 are both members of peptide growth factor families and are competent to act as signals in dorsal mesoderm induction (Dale *et al.*, 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995) or in dorsal-

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axis specification, respectively (Ku and Melton, 1993). *Xcat*-2 shows sequence homology with *nanos*, the posterior determinant in *Drosophila* (Mosquera *et al.*, 1993) and colocalizes with germ plasm (Forristall *et al.*, 1995), regions believed to contain the germ cell determinants (Ikenishi *et al.*, 1986).

During *Xenopus* oogenesis, there are at least two vegetal-mRNA localization pathways, one operating early in stage I oocytes and one late, in stage III oocytes (Forristall *et al.*, 1995). *Xcat*-2, *Xwnt*11, and *Xlsirts* specifically concentrate within a juxtanuclear structure unique to stage I oocytes called the mitochondrial cloud at a time when *Vg*1 is homogeneously distributed (Forristall *et al.*, 1995; Kloc and Etkin, 1995; Melton, 1987). *Xcat*-2 together with other RNAs is translocated to the future vegetal pole as a component of the cloud material. *Xcat*-2 localizes to a small discrete region of the vegetal cortex within germ plasm in stage II/III oocytes, where it remains throughout oogenesis and early embryogenesis (Forristall *et al.*, 1995). Heasman *et al.* (1984) have hypothesized that the mitochondrial cloud functions to localize germ plasm to the vegetal pole. *Vg*1 localizes later during stage III, with localization along the entire vegetal cortex completed by late stage IV. Therefore, *Vg*1 and *Xcat*-2 come to occupy different, but overlapping, cortical domains. These regional differences reflect differences in anchoring as well since *Vg*1 is rapidly released from the cortex during maturation, while *Xcat*-2 is retained (Pondel and King, 1988; Kloc and Etkin, 1994; Forristall *et al.*, 1995). In both the early and late pathways, transport and cortical anchoring represent independent steps as indicated by their different cytoskeletal requirements; microtubules in transport and microfilaments in anchoring (Yisraeli *et al.*, 1990).

Several localized RNAs have now been tested for their ability to correctly localize upon reintroduction into living cells (*Vg*1, Yisraeli and Melton, 1988; Mowry and Melton, 1992; *oskar*, Kim-Ha *et al.*, 1993; *bicoid*, Macdonald and Struhl, 1988; actin, Kislauskis *et al.*, 1994; MBP mRNA, Ainger *et al.*, 1993). The correct localization of these *in vitro* synthesized RNAs demonstrates that the RNA itself can determine selection for localization. For all localized mRNA examined, the 3′UTR has been found to contain information both required and sufficient for localization (St. Johnston, 1995). The results of fine deletion mapping of the *bicoid* (Macdonald *et al.*, 1993), *oskar* (Kim-Ha *et al.*, 1993), and actin (Kislauskis *et al.*, 1994) RLSs (RNA localization signals) suggest that the signal may consist of several redundant elements with different elements responsible for distinct steps in the localization pathway.

The *Xenopus* oocyte provides a powerful system in which to analyze mRNA localization. We have recently shown that *Xcat*-2 injected into stage I oocytes accumulates within the mitochondrial cloud (Zhou and King, 1996). The sequences required for localization to the cloud were mapped to a region within the 3′UTR. In this report, we show that *Xcat*-2 RNA is competent to localize to the vegetal cortex independent of the mitochondrial cloud and does so during the late (*Vg*1-like) pathway. Disrupting microtubules inhibits *Xcat*-2 transport, but does not affect cortical anchoring, results comparable to those described for *Vg*1 localization (Yisraeli *et al.*, 1990). A localization signal required and sufficient to direct *Xcat*-2 into the vegetal cortex was mapped to a small region in the 3′UTR. Localization is stage dependent. Although injected *Vg*1 and *Xcat*-2 RNAs are stable in stage VI oocytes, neither will localize in this cell, suggesting the localization machinery is no longer available.

MATERIALS AND METHODS

Oocyte isolation and culture. Adult frogs were anesthetized with 5 g/liter MS-222 (3-aminobenzoic acid ethyl ester) and a section of ovary was surgically removed. Oocytes were manually isolated in 1× modified Barth's saline–Hepes (MBSH; Gurdon, 1968) supplemented with 100 μg/ml of penicillin and 100 μg/ml of streptomycin. Stage VI (1.2–1.3 mm) or late stage III/early stage IV oocytes (0.5–0.7 mm) were injected with the synthetic transcripts to be tested and cultured in 0.5× Leibowitz medium supplemented with 1 m*M* L-glutamine, 1 μg/ml insulin, 15 m*M* Hepes (pH 7.8), 50 units/ml nystatin, 100 μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% frog serum containing vitellogenin (Wallace *et al.*, 1980; Yisraeli and Melton, 1988). Oocytes were cultured for up to 4 days at 20°C in a humidified chamber. Serum containing vitellogenin (green in color) was obtained from females that had been injected 3 weeks earlier with 0.4 ml, 10 mg/ml of estradiol-17β (Sigma) suspended in 1,3-propanediol (Wallace *et al.*, 1980; Yisraeli and Melton, 1988). The serum was inactivated at 56°C for half an hour, immediately aliquoted, and frozen at −80°C until used.

For the microtubule disruption experiments, injected oocytes were cultured as described above for 2 days. These oocytes were then placed on ice for 90 min and either cultured for an additional 4 days at 20°C or cultured overnight in medium containing 1 μg/ml of nocodazole dissolved in DMSO.

Oocyte extractions. After the culture period, whole oocytes were either immediately frozen at −80°C or fixed in a cold solution of 95% ethanol and 5% acetic acid at room temperature for a few minutes. Fixed oocytes were then cut into animal and vegetal halves. Total RNA was isolated from whole oocytes or halves by using a proteinase K method (Pondel and King, 1988). A LiCl precipitation step reduced glycoprotein contamination (Sambrook *et al.*, 1989). Typically, 10 oocytes or halves were collected for each time point.

Plasmid source and constructions. We have previously described the isolation of the *Xcat*-2 clone in pSPORT (Mosquera *et al.*, 1993). pNB40*Xcat*-2 tagged with a 50-nt *Xenopus* β-globin mRNA leader sequence and fused to the 5′ end of full-length *Xcat*-2 was a gift from Drs. R. Lehmann and C. Wang. The *Xenopus* β-globin (XβG), XβG-340/3′, and pSPT73XβG clones were gifts from Dr. K. Mowry and have been previously described (Mowry and Melton, 1992). ODC2 (ornithine decarboxylase) was used as an RNA loading control (Bassez *et al.*, 1990).

The deletion mutants used in this study were tagged with either the *Xenopus* β-globin 5′ leader sequence or a 413-nt luciferase fragment. The details of their construction have been described previously (Zhou and King, 1996). In addition, the *Xcat*-2 open reading frame (5′ORF) was isolated from pNB*Xcat*-2 as a *Mlu*I–*Bst*NI 470-bp frag-

ment and was ligated to a fragment containing the entire 3′UTR of β-globin for stability. Luc/3′UTRΔ1, Luc/3′UTRΔ2, Luc/3′UTRΔ3, and Luc/3′UTRΔ4 were constructed by ligations between the pT3/T7-luc3′ vector digested with *Bam*HI and *Apa*I (blunt-ended by Mung-bean nuclease) and 3′UTRΔ1, 3′UTRΔ2, 3′UTRΔ3, and 3′UTRΔ4 inserts cut with *Bam*HI and *Pvu*II (blunt-end cutter). All mutant constructs are diagramed in Fig. 5.

In vitro RNA transcription. The templates and procedures for *in vitro* transcription were as previously described in Zhou and King (1996). Luc/3′UTR, Luc/3′UTRΔ1, Luc/3′UTRΔ2, Luc/3′UTRΔ3, and Luc/3′UTRΔ4 were linearized with *Xho*I and transcribed with T3 RNA polymerase to produce 870, 730, 630, 730, and 600-nt transcripts. Polyadenylation of ^{35}S-labeled transcripts was carried out following the procedures of Rusconi and Flick (1993) by using recombinant yeast poly(A) polymerase (Drummond *et al.*, 1985).

Probes and RNase protection. Probe construction and RNase protection assays were performed exactly as presented in Zhou and King (1996) based on methods described in Melton *et al.* (1984). The 210-nt probe generated by *Hin*dIII digestion resulted in a protected fragment of 144 nt for endogenous *Xcat*-2 and a fragment of 194 nt for injected *Xcat*-2. The probe for Xβ G-340/3′ transcripts was transcribed from linearized pSPT73Xβ G5′ plasmid by SP6 RNA polymerase (Mowry and Melton, 1992). The antisense riboprobes for the 3′UTRΔ1, 3′UTRΔ2, 3′UTRΔ3, and 3′UTRΔ4 transcripts were used for RNase protection. The probe for the 3′UTR transcripts was the same as the one for 3′UTRΔ1 (Fig. 5). The LucP clone was generated by ligating the *Eco*RV–*Eco*RI fragment (1419–1871 of luciferase insert) from pT3/T7-luc3′ to the pT3/T7 vector cut with *Eco*RV and *Eco*RI. The antisense riboprobe was produced by cutting the probe clone with *Xho*I and transcribing with T7 RNA polymerase. The resulting probe was about 460 nt in size and protected at 371-nt fragment in the luciferase tag. The probe for ODC (ornithine decarboxylase) protected a 90-nt fragment (Bassez *et al.*, 1990).

In situ hybridization. The procedures followed for nonradioactive *in situ* hybridization have been previously described (Oberman and Yisraeli, 1995; Harland 1991). Oocytes were defolliculated by treatment with collagenase (Mosquera *et al.*, 1993) and Dumont stages III, IV, and VI isolated by hand. Oocytes were fixed in 0.25% chromium trioxide in 95% ethanol and 5% acetic acid on ice for at least 1 hr. After dehydration and infiltration with paraplast, oocytes were embedded and sectioned at 7 μm. Sections were dried at room temperature, baked at 58°C for 45 min, and then incubated overnight at 37°C. Sections were dewaxed in xylene and rehydrated in a graded ethanol series. After fixation with 4% paraformaldehyde in 1× PBS for 20 min, slides were incubated at 37°C in 0.1 *M* Tris, pH 7.5, 0.01 *M* EDTA containing 1.5 μg/ml proteinase K (purchased in solution from Boehringer Mannheim) for 5 min. After rinses in 0.2 *M* HCl and 0.1 *M* triethanolamine, pH 8.0, with 0.25% acetic anhydride, slides were dehydrated and air dried. PEP–PEN (Electron Microscopy Science) was used to create a water-impermeable barrier around the sections. All subsequent steps were carried out in a humidified chamber. After drying at room temperature, the hybridization solution was added to each slide and the slides were placed at either 58°C for *Vg*1 or 45°C for *Xcat*-2 hybridization. After 1 hr of prehybridization, hybridization solution with denatured probes was added and hybridization allowed to continue overnight. At the end of the hybridization period, slides were washed in 2× SSPE, 0.1% CHAPS for 30 min at room temperature, treated with RNase A (20 μg/ml) in 4× SSPE with 0.1% CHAPS for another 30 min, and finally washed in 0.1× SSPE at room temperature for 5 min. Prior to immunodetection of the probe, slides were washed in 1× PBS, 0.1% Tween 20 for 10 min followed by PBT (1× PBS, 2 mg/ml BSA, 0.1% Triton X-100) for 15 min. Immunodetection of the probe was carried out exactly as described in Zhou and King (1996), with BM purple AP as substrate (BMB). Samples were then dehydrated, treated with xylene, and mounted in Paramount (Sigma).

^{35}S-labeled mRNA autoradiography. Stage IV oocytes were injected with ^{35}S-labeled, capped, and polyadenylated transcripts (sp act $\sim 5 \times 10^7$ dpm/μg) and the oocytes cultured (Krieg *et al.*, 1984; Rusconi and Flick, 1993; Drummond *et al.*, 1985). At each time point, oocytes were collected and fixed in 0.25% chromium trioxide in 95% ethanol and 5% acetic acid on ice for at least 1 hr. After dehydration and infiltration with paraplast, oocytes were embedded and sectioned at 7 μm. Sections were dried overnight at 45°C, dewaxed by two incubations in xylene (10 min each), and rehydrated through a graded series of ethanol starting at 30%. Slides were processed for autoradiography and exposure times were for 1 to 3 weeks (Zhou and King, 1996).

RESULTS

Injected Xcat-2 Transcripts Concentrate in the Vegetal Hemisphere of Stage IV Oocytes

Endogenous *Xcat*-2 mRNA accumulates within the mitochondrial cloud sometime during stage I of oogenesis, at a time when *Vg*1 RNA is uniformly distributed in the oocyte (Forristall *et al.*, 1995; Melton, 1987; Yisraeli and Melton, 1988). The cloud breaks up and *Xcat*-2 containing fragments move to the vegetal cortex during stage II. Is the mitochondrial cloud a large transport complex essential for *Xcat*-2 localization to the vegetal pole or, like *Vg*1, is *Xcat*-2 capable of localization later, independent of the mitochondrial cloud? To answer this question, early stage IV oocytes were injected equatorially with full-length *Xcat*-2 RNA transcripts, and localization monitored by RNase protection assays of RNAs obtained from animal (A) or vegetal (V) halves. The amount of *Xcat*-2 injected was twice the endogenous amount (100 pg). Because approximately half (40 to 70%) of the *Xcat*-2 transcripts will be degraded after 4 days of culture, the injected amount represents a modest increase over endogenous levels. This loss is consistent with degradation rates reported for injected *Vg*1 (Yisraeli and Melton, 1988). A small number of oocytes were assayed 1 to 2 hr after injection (Day 0), and as expected, the majority of injected RNA was randomly distributed (Fig. 1, compare A and V lanes). Endogenous *Xcat*-2 levels served as an internal control for oocyte viability as well as for indicating accurate cutting of A/V halves.

The RNase protection assays show that the injected *Xcat*-2 begins to accumulate within the vegetal half by the end of Day 1 and that this process is complete by Day 3 or 4 of culture (Fig. 1). The pattern observed is that which is expected if degradation of the injected transcripts is the same in both halves over the culture period, and *Xcat*-2 is being

moved out of the animal half into the vegetal half. The findings suggest that *Xcat*-2 can localize independently of the mitochondrial cloud and further that the components of the late pathway do not represent a rate limiting step in the localization process.

Ectopic Xcat-2 Localizes into the Cortex of Stage IV Oocytes

To more closely compare localization events between the endogenous *Vg*1 and injected labeled *Xcat*-2, we followed their movement to the vegetal cortex over time by *in situ* hybridization and autoradiography, respectively. We analyzed over 50 early and late stage III's and early and late stage IV's for this study. *Vg*1 localization is first detected in stage III and is accomplished by late stage IV (Figs. 2A–2D). In late stage II/early III's, *Vg*1 is uniformly distributed (Forristall *et al.*, 1995; Melton, 1987) (not shown). The first sign of asymmetry was a withdrawal of *Vg*1 from the animal pole cortical region in stage III's. Next, the *Vg*1 signal was observed concentrated around the nucleus (Figs. 2A and 2B). Significant levels of *Vg*1 have already accumulated within the vegetal subcortex in stage III. In late stage III's, *Vg*1 signal is detected exclusively on the vegetal pole side of the nucleus. The signal is confined to a trapezoid-shaped zone stretching from an apex at the basal side of the nucleus down to a broader cortical domain centered at the vegetal pole (Figs. 2B and 2C). The complexes become concentrated in the cortical area (Fig. 2D). The final pattern in late IV's reveals *Vg*1 accumulated in the cortex stretching from the vegetal pole to the marginal zone, with signal above this region indicating that some *Vg*1 fails to enter the cortex, even by stage VI. This localization pattern is most consistent with a model in which endogenous *Vg*1 is localized by active transport, in agreement with results obtained by Melton and co-workers (Melton, 1987; Yisraeli and Melton, 1988; Mowry and Melton, 1992).

Injected radiolabeled *Xcat*-2 mRNA localizes into the vegetal cortex as was observed for endogenous *Vg*1, but not as efficiently. Here we have looked at 24-hr time periods and followed *Xcat*-2 localization by autoradiography in sectioned albino or bleached wildtype oocytes. One day after injection, *Xcat*-2 appears uniformly distributed over the oocyte; the signal present as fine silver grains (Fig. 2E). In some cases, such as the one shown, a bias in *Xcat*-2 signal was already apparent. By Day 2, a distinct polarity in the *Xcat*-2 signal was observed. Label had accumulated around the nucleus, mostly on one side, and within the subcortex at one pole. By Day 3, more labeled *Xcat*-2 was observed in a trapezoid-shaped zone similar to that observed for endogenous *Vg*1 (Figs. 2F, 2G, and 3). *Xcat*-2 had also accumulated into the vegetal subcortex (Fig. 2G). By Day 4, *Xcat*-2 is highly concentrated within the vegetal cortex, but a significant amount remains within the vegetal zone (Fig. 2H). Clearly, injected *Xcat*-2 is less efficient at cortical localization than is endogenous *Vg*1 RNA. Transcripts which fail

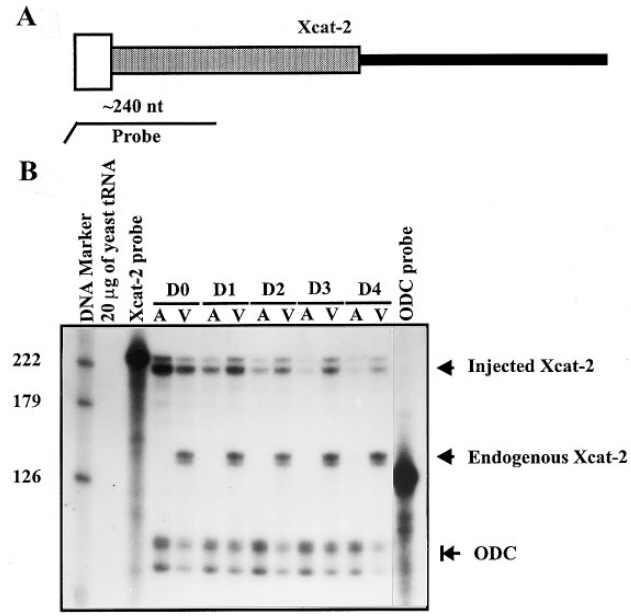


FIG. 1. *Xcat*-2 RNA microinjected into stage IV oocytes localizes to the vegetal half. (A) Schematic of the construct used to make transcripts for injection. Open box represents the 5′ leader sequence of *Xenopus* β-globin mRNA, ~50 bp long. Filled box represents the 5′ leader and coding region of *Xcat*-2, ~400 bp long. Thick line represents the 3′ UTR of *Xcat*-2, ~410 bp long. The region protected in an RNase protection assay and the size of the probe are indicated. (B) The concentration of *Xcat*-2 in animal (A) or vegetal (V) halves on Day 0 (D0) through Day 4 (D4) of culture was assessed by RNase protection. 100 pg of capped *Xcat*-2 transcript was injected into stage IV oocytes. Total RNA from two oocyte halves were loaded on each lane. In this example, localization appears complete after Day 3. Endogenous *Xcat*-2 served as an internal control for oocyte viability and accurate cutting of A/V halves. ODC (ornithine decarboxylase) was included as a control for RNA loading. Yeast tRNA served as a control for nonspecific hybridization. The labeled probes for ODC and *Xcat*-2 are shown as well as DNA markers in bp.

to enter the cortex most likely represent degraded and/or untransported *Xcat*-2. In contrast, injected *Xenopus* β-globin RNA, a nonlocalized RNA, fails to demonstrate any of these localization steps (Fig. 5Aa). Because ectopic *Xcat*-2 mRNA is competent to localize into the vegetal cortex, we conclude that it must contain cis-localization elements which can be recognized by the localization machinery operating in the late pathway.

Translocation Is Dependent on Intact Microtubules

Previous studies have used nocodazole or colchicine to depolymerize microtubules and have demonstrated a role for these structures in the active transport of *Vg*1 RNA

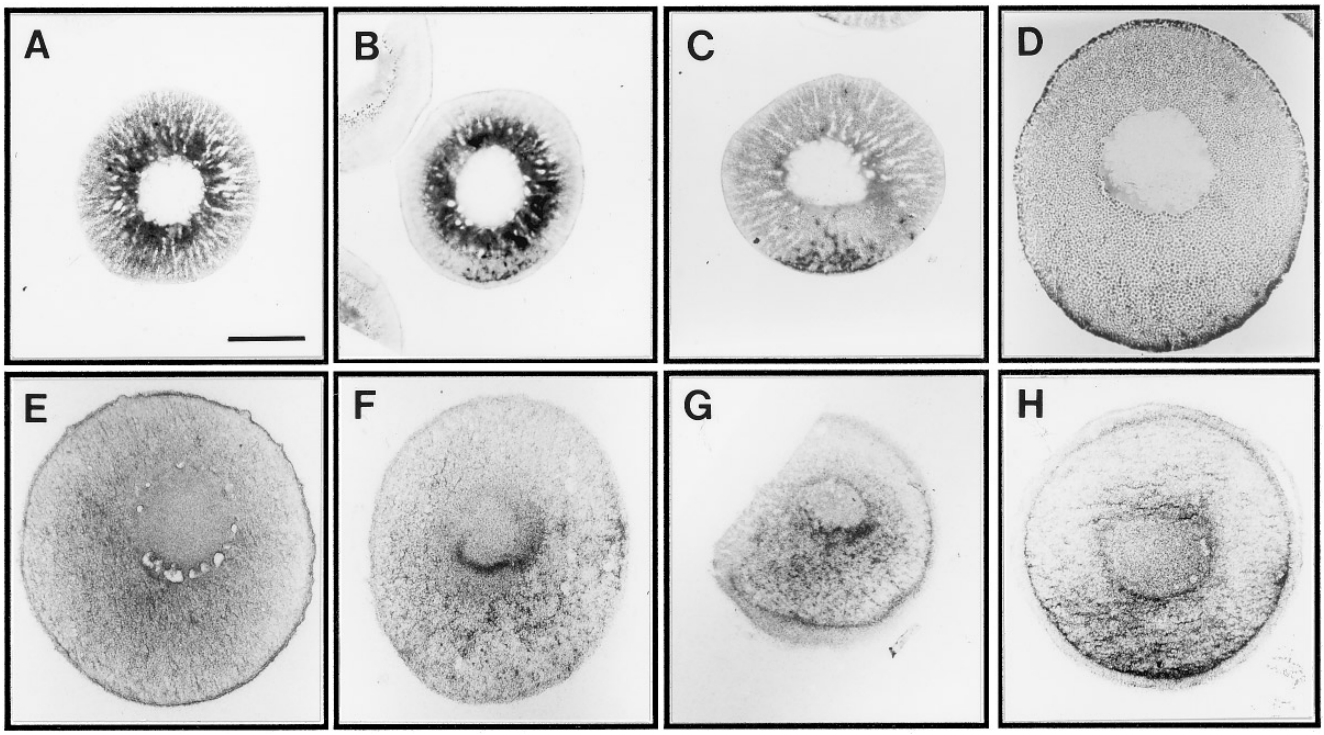


FIG. 2. Injected *Xcat*-2 mRNA uses the same localization pathway as endogenous *Vg*1 in stage IV oocytes. (A–D) *In situ* hybridizations showing *Vg*1 localization using DIG antisense probes. (A) Section of a stage III oocyte. *Vg*1 moving toward the nucleus. (B) Section of a stage III oocyte. Endogenous Vg1 starts to concentrate around the nucleus and moves toward one pole (the vegetal pole). (C) Section of an early stage IV oocyte; more *Vg*1 appears near the vegetal cortex. Signal is very distinctive. (D) Section of late stage IV oocyte. Majority of the endogenous *Vg*1 is found in the vegetal cortex. (E–H) Autoradiograms showing localization of injected ^{35}S-labeled, capped, and polyadenylated *Xcat*-2 RNA after injection into early stage IV oocytes. (E) After 1 day of culture, silver grains were observed throughout the cytoplasm with a concentration on the basal side of the nucleus and into the vegetal half. (F) After 2 days, grains were observed concentrated mainly on the basal side of the nucleus and within aggregates between the nucleus and the cortex. (G) After 3 days, *Xcat*-2 was observed in the cortex. (H) After 4 days, the majority of the label was in the oocyte cortex, although a significant amount of *Xcat*-2 remains in the vegetal yolky area. Bar in A for A–H, 130 μm.

(Yisraeli *et al.*, 1990). We asked if ectopic *Xcat*-2 localization in stage IV oocytes was also microtubule dependent. Oocytes placed on ice for 90 min have all their microtubules, including those in the cortex, completely depolymerized, as shown by confocal immunofluorescence microscopy (Gard, 1991, 1993). Upon warming to room temperature, microtubules rapidly regrow from the nucleus in 30 to 60 min, but a complete return to normal microtubular organization takes 1 to 2 days in stage IV or VI oocytes (Gard, 1991). Microfilaments and intermediate filaments were not affected by exposure to low temperature (Gard, 1991, 1993). Oocytes were cold treated either shortly after injection of ^{35}S-labeled *Xcat*-2 transcripts or 2 days later, after *Xcat*-2 transport complexes have formed and a portion are in the vegetal cortex (Fig. 2F). In the first group, *Xcat*-2 localization was monitored at the end of a 2-day culture period by RNase protection analysis of A/V halves. In these oocytes, *Xcat*-2 remained uniformly distributed compared to untreated controls (data not shown). These results suggest that transport requires not just intact microtubules, but a specific organization of microtubules.

In the second group, cold treated after 2 days of culture, oocytes were cultured at 20°C in either the presence or the absence of nocodazole. In the oocytes where microtubules were completely depolymerized and allowed to reform at room temperature, *Xcat*-2 localization was delayed by 2 days (Fig. 3). It now required 6 days to achieve the degree of vegetal accumulation that was observed after 4 days in the untreated control oocytes. This time period corresponded to the time it takes for normal microtubular organization to reestablish itself. In contrast, cortical anchoring was not sensitive to cold treatment, as *Xcat*-2 remained in the cortex of stage IV or VI oocytes kept on ice for 2 hr (data not shown). Therefore, intact microtubules are required for *Xcat*-2 to accumulate into the vegetal cortex, but not for its retention within the cortex. These results are in complete

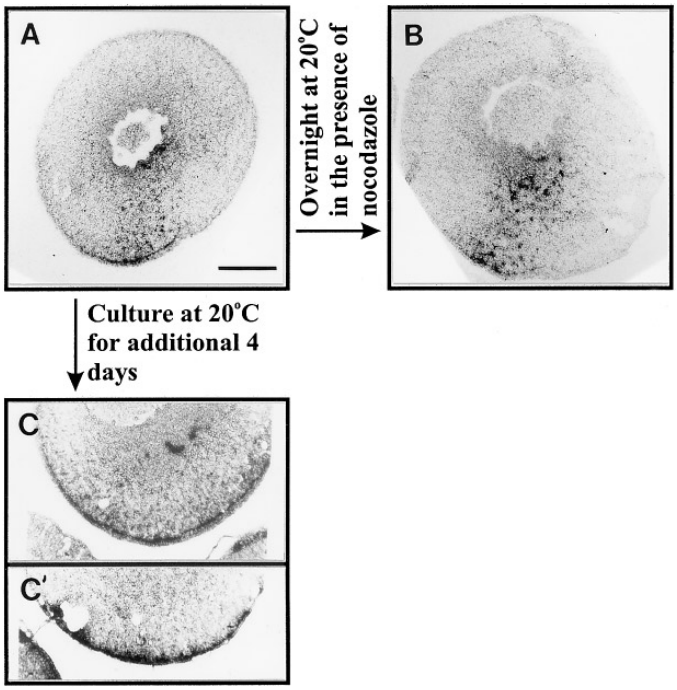


FIG. 3. The translocation process of *Xcat*-2 in stage IV oocytes is microtubule dependent and the integrity of *Xcat*-2 translocation complexes is microtubule independent. Autoradiograms of IV oocytes injected with ^{35}S-labeled, capped, and polyadenylated *Xcat*-2 transcripts. (A) Oocyte cultured for 2 days and then placed on ice for 90 min. (B) The same as in (A) but then cultured in the presence of 1 μg/ml of nocodazole at 20°C for 24 hr. Note that the *Xcat*-2 transcripts remain asymmetrically distributed and within complexes. (C) The same as in (A) and then cultured at 20°C without nocodazole for an additional 4 days. Virtually all of the injected transcripts reach the vegetal cortex. (C′) Another example of an oocyte treated as in C. Bar in A for A–C′, 130 μm.

agreement with those obtained using nocodazole and cytochalasin to study *Vg*1 localization and cortical anchoring and further show that *Xcat*-2 is competent to localize in a *Vg*1-like late pathway (Yisraeli *et al.*, 1990).

Interestingly, in cold-treated oocytes allowed to recover, a much higher proportion of the injected *Xcat*-2 consistently entered the cortex than the controls kept at 20°C (compare Fig. 3C with Fig. 2H). Furthermore, the accumulation of *Xcat*-2 at the basal side of the nucleus (Figs. 2F and 2G), the most obvious difference between the *Vg*1 and *Xcat*-2 patterns of localization is abolished (Fig. 3C). In stage III and IV oocytes, this perinuclear region is yolk free and stains strongly for microtubules, especially on the basal side (Gard, 1991). These observations suggest that injected transcripts are more efficiently incorporated into the oocyte's transport system if microtubules are depolymerized and allowed to reassemble in their presence. We postulate that *Xcat*-2 accumulates at this site because the next step in transport involves association with microtubules and is rate limiting for injected RNAs.

In the absence of microtubules, the polarized distribution of *Xcat*-2 during translocation might be expected to be lost. However, *Xcat*-2 did not relocate after cold treatment, indicating that *Xcat*-2 was not "free" to diffuse in the oocyte (compare Fig. 2E with Figs. 3A and 3B). One possibility was that the *Xcat*-2 complexes reassociated with the repolymerized microtubules within 30–60 min after warming and this prevented the complexes from moving. To test this hypothesis, cold-treated oocytes were allowed to reach room temperature in the presence of nocodazole and were cultured for an additional 24 hr in this drug. Under these conditions where microtubule repolymerization was inhibited, *Xcat*-2 complexes remained intact and in the same relative position as was observed before cold treatment (Figs. 3A and 3B). These results imply that transport particles are associated with another cytoskeletal system and/or are simply too dense and large to diffuse within the oocyte.

The 3′UTR of Xcat-2 Is Required and Sufficient for the Localization in Stage IV Oocytes

As shown in Figs. 1 and 2, *Xcat*-2 alone contained the information required for localization. In order to map the putative localization signal in *Xcat*-2, we first tested the 3′UTR to determine if it was both required and sufficient for vegetal localization. Two deletion mutants were made that divided *Xcat*-2 into the coding region and the 3′UTR. Each deletion mutant was tagged at the 5′end with a 50-bp globin leader sequence. The *Xcat*-2 coding region by itself proved to be unstable in oocytes; therefore, the 3′UTR of *Xenopus* β-globin was added (Fig. 4A). The transcripts derived from these mutants were injected into stage IV oocytes and the oocytes cultured for 4 days. The 3′UTR localized as well as full-length *Xcat*-2, whereas the 5′ORF was localization incompetent (Fig. 4B). Therefore, the 3′UTR was required for RNA stability and vegetal localization.

To demonstrate if the 3′UTR was sufficient for localizing *Xcat*-2, the 400-nt coding region of *Xcat*-2 was exchanged for a similarly sized fragment containing the ORF of luciferase (Fig. 4A, Luc/3′UTR). Figure 4B shows that the *Xcat*-2 3′UTR was sufficient to localize luciferase to the vegetal half of stage IV oocytes. The 3′UTR was also sufficient for vegetal cortical localization as radiolabeled Luc/3′UTR injected into normally pigmented oocytes concentrated within the vegetal cortex in a pattern indistinguishable from full-length *Xcat*-2 (Figs. 2G and 2H) (data not shown).

The Xcat-2 3′UTR Contains at Least Two Localization Signals

The signal required for *Xcat*-2 to enter the mitochondrial cloud in stage I oocytes has been mapped to a 250-nt region in the 3′UTR (Zhou and King, 1996). It was of interest to determine how that signal related to the minimal sequence

required for vegetal localization in stage IV oocytes. To that end, four deletion mutations within the 3′UTR of *Xcat*-2 were constructed. In addition, to show the sufficiency of these mutants in localizing RNA to the vegetal half, the 400-nt coding region of luciferase was ligated to each fragment. To keep the length of the 3′UTR constant between the mutants, 200 nt of vector sequence was included at the 3′ end. In subsequent experiments, we found that the length of the 3′UTR was not critical, as transcripts behaved the same in the localization assay with or without the 200-nt vector sequence. The assay was done in two parts. Oocyte injection of radiolabeled chimeric transcripts tested for their ability to localize cortically. Figure 5A shows typical examples of constructs which were scored as unlocalized including *Xenopus* β-globin RNA and 5′ORF/XβG3′UTR (a), weakly localized (b), or strongly localized (c) after 2 days of culture. Although oocytes were commonly observed that showed intermediate levels of localization, weak localization as shown in (b) represents the worst case that was scored as positive. In fact, no oocytes were observed whose pattern fell between the weak and the nonlocalized condition. Mutant 3′UTRΔ1, containing 200 nt proximal to the ORF was sufficient for localization, but localized only half as well as the full-length 3′UTR (Fig. 5C). The failure of 3′UTRΔ2 to localize eliminated any sequences in the last 200 nt as being sufficient. In 3′UTRΔ4, the middle 200 nt of the 3′UTR with overlapping sequences from 3′UTRΔ2 was tested and proved deficient. 3′UTRΔ3 tested the first 150 nt and the last 120 nt of the 3′UTR and was found to localize as efficiently as the full-length 3′UTR. These results are summarized in Fig. 5B and show that a sequence of 150 nt immediately adjacent to the ORF and additional sequences 120 nt at the end of the 3′UTR are required and sufficient for vegetal and cortical localization equivalent to the entire 3′UTR.

The RNase protection assay tested competency of the chimeric sequence to accumulate into the vegetal hemisphere and provided a means to quantitate localization using the PhosphoImager. Results from two series of injections are summarized in Fig. 5C, which shows the percentage change in transcript levels for each mutant construct in both the animal and vegetal halves after 4 days of culture. Deletion mutants were scored as localization positive that showed 80% (±15%) of the remaining injected transcript in the vegetal half. Deletion mutants were scored as failing to localize if 50% (±5%) remained in either half. In no instance was a mutant transcript capable of vegetal but not cortical localization (Fig. 5B).

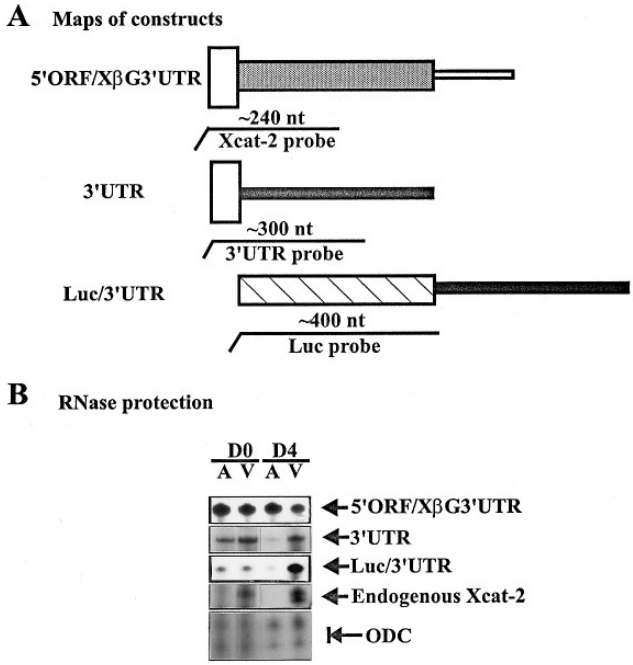
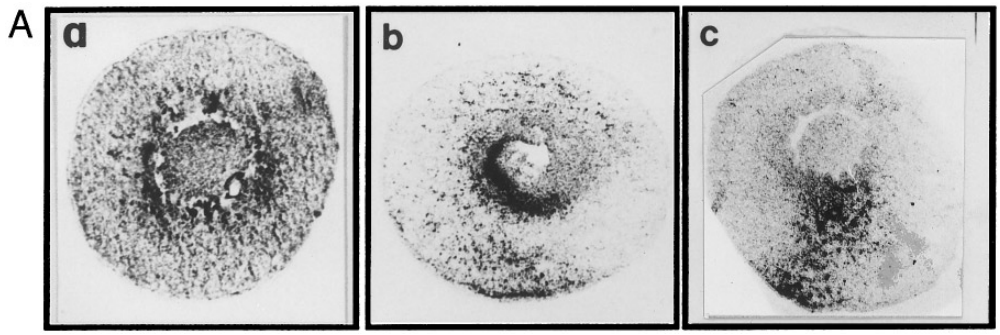


FIG. 4. The 3′UTR of *Xcat*-2 is required and sufficient for *Xcat*-2 localization in stage IV oocytes. (A) Diagrams of the cDNA constructs used for making injected transcripts. The probes for the RNase protection assays are shown by thin lines with their sizes indicated. Open boxes, the 5′ leader sequence of *Xenopus* β-globin (~50 bp). Shaded box, the 5′ leader and coding sequence of *Xcat*-2 (~400 bp). Thick line, the 3′UTR of *Xcat*-2 (~410 bp). Striped box, a portion of luciferase coding region. (B) An RNase protection assay for localization of *Xcat*-2 5′ORF and 3′UTR after Day 0 (D0) and Day 4 (D4) of culture. Total RNA from animal (A) or vegetal (V) halves was assayed. The 3′UTR of *Xcat*-2 and the luciferase-tagged 3′UTR of *Xcat*-2 are localized to the vegetal half of oocytes, while the 5′ORF/XβG3′UTR is not. (D0) One-quarter of an oocyte equivalent was loaded; (D4) two oocyte equivalents were loaded. ODC probe was included as a control for RNA loading.

Injected RNAs Do Not Localize in Stage VI Oocytes

For both *Vg*1 (Melton, 1987) and *Xcat*-2 (Forristall *et al.*, 1995), RNA localization occurs during specific stages. Is the availability of the RNA transport machinery limited to specific stages or can these RNAs be localized after stage IV? To address this question, *in vitro* synthesized *Xcat*-2 RNA and the *Vg*1 localization element (XβG-340/3′) were co-injected (50 pg each) into hand-dissected stage VI oocytes. After 4 days, RNase protection assays on animal and vegetal halves were carried out. Neither the injected *Xcat*-2 nor the *Vg*1 localization element localized to the vegetal half of VI oocytes, but instead, remained uniformly distributed in the oocyte (Fig. 6B). Both *Vg*1 and *Xcat*-2 failed to localize, indicating the specificity of *Xcat*-2 localization in the stage IV oocyte. These results suggest that stage VI oocytes are deficient in some component(s) of the localization machinery.

Is RNA that fails to localize less stable than localized RNA? Rapid degradation of such RNAs would prevent the potentially harmful situation of mislocalized proteins in a cell. To address this question, we compared the stability of



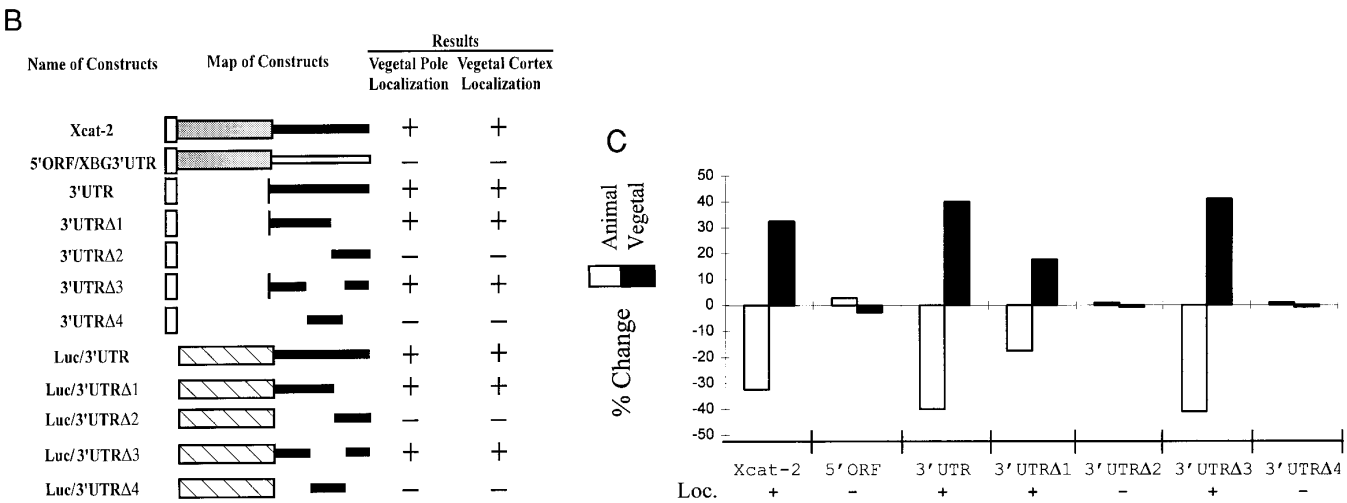


FIG. 5. A cis-localization signal located within the 3′UTR is necessary and sufficient for *Xcat*-2 mRNA localization. (A) Sections of oocytes showing examples of injected deletion mutants that failed to localize (a), localized weakly (b), or localized strongly (c). (B) Schematic of the series of the mutant transcripts tested for localization in stage IV oocytes. Deletions are indicated by interruptions in the lines. After the culture period, localization of the injected transcripts was scored as negative (−) or positive (+). Failure to localize was always absolute. Oocytes were scored as positive that ranged from what is depicted in (b) and (c). The vegetal half localizations were monitored by RNase protection assays. Localization to the vegetal cortex was monitored by autoradiography (radiolabeled transcripts) or by *in situ* hybridizations (DIG-labeled probe) (see Fig. 4A and Materials and Methods for details). (C) Histogram showing quantitation of the RNase protection assays from two series of experiments using the PhosphoImager (Molecular Dynamics). Results are plotted for each mutant transcript as the percentage gained or lost in the animal or vegetal halves at the end of the culture period relative to the total remaining.

Xcat-2 and *Vg*1 transcripts in stage IV and VI oocytes by measuring the signal remaining after each day by RNase protection. Assays were quantified using the PhosphoImager. The results show no significant difference in the stability of these RNAs at the two stages examined. In both cases, approximately 70% of the initial signal remained after 2 days and between 40 to 60% after 4 days in culture. We conclude that for *Vg*1 and *Xcat*-2 RNAs, there is no correlation between message stability and the localized condition.

DISCUSSION

Although *Xcat*-2 and *Vg*1 share a common ooplasm in stage I oocytes, *Xcat*-2 localizes to the cortex early in stage II oocytes as a component of the germ plasm and mitochondrial cloud, while *Vg*1 localizes later during stage III as an RNP complex that requires microtubules for transport (Yisraeli *et al.*, 1990; Forristall *et al.*, 1995; King, 1995). Here we have shown that *Xcat*-2 RNA is competent to localize to the vegetal cortex independently of the mitochondrial cloud in stage IV oocytes. *Xcat*-2 appears to use the late pathway based on the following similarities with *Vg*1 localization. *Xcat*-2 localization required intact microtubules as cold or nocodazole treatment resulted in a cessation of transport whether treatment was just after injection or 2 days later after transport was well initiated (Fig. 3). Once in the cortex, *Xcat*-2 occupied the broader domain characteristic of *Vg*1 (compare Figs. 2D and 2H) and not the more limited one observed for endogenous *Xcat*-2 as determined

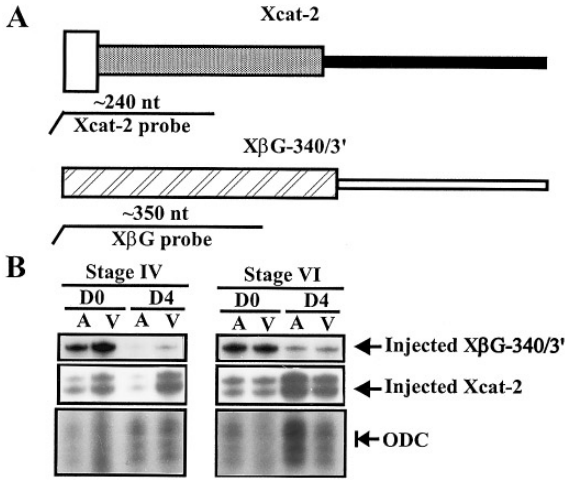


FIG. 6. Injected *Xcat*-2 and *Vg*1 transcripts localize in stage IV oocytes but not in stage VI oocytes. (A) A diagram of the cDNA constructs used in making *Xcat*-2 or XβG-340/3′ transcripts. Striped box indicates the coding region of *Xenopus* β-globin (~490 bp). Open thick line indicates the *Vg*1 localization element (340 bp). Thin line indicates the probe for RNase protection assays (~350 bp). *Xcat*-2 construct is the same as that indicated in Fig. 1A. (B) An RNase protection assay with total RNA isolated from animal (A) or vegetal (V) halves of the injected oocytes. 50–100 pg each of *Xcat*-2 and XβG/340 transcript was injected into each oocyte. Ten oocyte halves were collected for each time point. For Day 0 (D0) one-quarter of an oocyte equivalent was loaded. For Day 4 (D4), two oocyte equivalents were loaded. ODC (ornithine decarboxylase) probe was included as a control for RNA loading. Note that neither injected *Vg*1 nor *Xcat*-2 transcripts localized in stage VI oocytes.

by measuring the diameter of the two cortical domains (Forristall *et al.*, 1995). This suggests that the transport system defines the cortical domain that the RNA will occupy. Finally, similar to *Vg*1, intact microtubules were not required to maintain *Xcat*-2 in the cortex (Yisraeli *et al.*, 1990; Fig. 3). These results indicate that *Xcat*-2 RNA must contain the cis-localization elements which are recognized by the transport and anchoring machinery operative in the late pathway. Furthermore, these components must be present in excess to the extent that ectopic *Xcat*-2 was accommodated by the transport machinery.

Endogenous *Vg*1 localization was more complete than was observed for the injected *Xcat*-2. There are several explanations for this. A portion of the unlocalized signal may represent reincorporated [^{35}S]UTP generated from degraded *Xcat*-2 into other endogenous RNAs. *Xcat*-2 localization elements may not be as efficient at transport as are the *Vg*1 signals. It is possible that the late stage localization process is initiated from inside the nucleus and, if so, injected *Xcat*-2 would not be exposed to any nuclear factors that may be required for efficient localization. Finally, injected *Xcat*-2 may not compete efficiently at the level of transport particle assembly. We favor the later explanation because injected *Xcat*-2 could be efficiently transported to the cortex like endogenous Vg1 if microtubules were first depolymerized and then allowed to repolymerize in the presence of exogenous *Xcat*-2 transcripts (Fig. 3C).

The organization of microtubules in the oocyte is consistent with its purported role in late RNA transport and suggests that the basal side of the GV may be a special region involved in transport. In stage IV oocytes, microtubules are concentrated in a yolk-free area surrounding the GV, with the highest concentration on the vegetal or basal side. Microtubules radiate between the dense meshwork of cortical microtubules and the surface of the GV (Gard, 1991). The GV appears to be the only MTOC in oocytes (Gard, 1991), arguing that microtubules are predominately organized with plus ends anchored in the cortex and minus ends around the GV. However, it is possible that minus ends of microtubules are also at the vegetal cortex (Gard, 1994).

We observed an accumulation of injected *Xcat*-2 RNA on the basal side of the nucleus. This observation may be significant in as much as it indicates a rate limiting step in the translocation process. Endogenous *Vg*1 accumulation at this site is not commonly observed, although examples can be found in the literature (Melton, 1987; Yisraeli *et al.*, 1990). As a working hypothesis, we propose that individual RNPs move along microtubules using a minus end directed (dynein-like) motor to arrive at the basal side of the GV (Yisraeli *et al.*, 1990; this report). Transport complexes most likely would interact with plus end-directed motors, like kinesin. It is possible that RNAs can be efficiently targeted to the vegetal cortex by restricting the site of assembly and the type of motor allowed to associate with the transport complex. In this view, there is no need to invoke special ''receptors'' in the vegetal cortex for RNAs localized in the late pathway, but simply that transport complexes contain cytoskeletal binding proteins.

Deletion mapping of *Xcat*-2 has defined a sequence of 150 nt immediately adjacent to the ORF and an additional sequence 120 nt at the end of the 3′UTR that are both required and sufficient for vegetal cortical localization in the late pathway. These sequences were equivalent to the entire 3′UTR in terms of localization activity. Direct comparison of these sequences with the *Vg*1 340-nt localization signal (Mowry and Melton, 1992) did not uncover any significant homologies or related secondary structures. However, additional experiments would need to be done to reveal any functional secondary structure(s) of significance. Although, the localization assays tested for both vegetal accumulation and cortical localization, *Xcat*-2 mutants that were competent to move into the vegetal hemisphere, but were unable to enter the cortex, were never observed. One interpretation of these findings is that the transport particle contains all the components required for cortical anchoring as well as translocation. If this were true, it would also help to explain why *Xcat*-2 RNA is competent to use the late localization pathway although it normally localizes as a

component of the fragmented mitochondrial cloud during stage II. That is, the same RNA sequences are required for transport and anchoring. The endogenous *Xcat*-2 RNA complex is capable of cortical anchoring; therefore, it also contains the information for vegetal localization. Such a dual function has been described for staufen protein in *Drosophila* where it is required for particle formation and anchoring of *bicoid* at the anterior pole (Ferrandon *et al.*, 1994). However, we cannot rule out the possibility that within the localization signal there are different elements responsible for the different steps of localization. Fine deletion mapping of the signal might reveal such elements as have been identified for *Drosophila bicoid* (Macdonald *et al.*, 1993).

We have previously found that *Xcat*-2 injected into stage I oocytes will localize to the mitochondrial cloud, whereas *Vg*1 fails to do so (Zhou and King, 1996). Clearly, the components of the late localization pathway are not available in stages I, II, or VI. The mitochondrial cloud localization signal mapped to the proximal 250 nt of the 3′UTR, a region larger than the 150-nt proximal piece involved in the vegetal cortex signal (Zhou and King, 1996). Although the significance of such an observation is unknown, it is tempting to speculate that two separate RNA pathways evolved during the course of *Xenopus* oogenesis. One pathway is specialized for the transport of germ plasm by way of the mitochondrial cloud and occurs early to ensure the segregation of the germ cell lineage. The other, late, pathway would serve as the more general transport system for localizing RNAs involved in somatic cell differentiation. Further characterization of these two pathways should yield insights into the mechanism involved in RNA transport.

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